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13. ABSTRACT *(Maximum 200 Words)*

Immediate-early response genes play a role in both survival and death. This concept award proposed that the opposing biological activities of the immediate-early response genes were regulated by their subcellular localization. That is, the mitogenic activities of these genes occur in the nucleus through their target gene regulation, whereas their apoptosis-inducing effect occurs in the cytoplasm through their modulation of mitochondrial activities. We have studied the subcellular localization of immediate-early response genes TR3 and c-myc in response to apoptotic stimuli in breast cancer cells by confocal microscopy analysis. Our results demonstrated that TR3 migrated from the nucleus to mitochondria in response to apoptotic stimuli in breast cancer cells, while c-myc localized exclusively in the nucleus under the same treatments. Our results, therefore, suggest that inducing TR3 mitochondrial localization may be an attractive approach to induce breast cancer cell apoptosis. Thus, orphan receptor TR3 may be used as a molecular target for developing agents that induce breast cancer cell apoptosis.

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INTRODUCTION

This concept award intended to provide evidence that immediate-early response genes, such as orphan receptor TR3 (nur77) and c-myc, can be used as molecular targets for developing agents that induce breast cancer cell apoptosis. Immediate-early response genes play a role in both survival and death (1, 2). This concept award proposed that the opposing biological activities of the immediate-early response genes were regulated by their subcellular localization. That is, the mitogenic activities of these genes occur in the nucleus through their target gene regulation, whereas their apoptosis-inducing effect occurs in the cytoplasm through their modulation of mitochondrial activities. The concept would be tested by confocal microscopy analysis of subcellular localization of immediate-early response genes fused with green fluorescent protein (GFP) in response to death stimuli in breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

Mitochondrial targeting of TR3 in breast cancer cells. It was believed that the immediate-early response genes function as transcriptional factors to regulate expression of genes responsible for cell survival and death (1,2). We recently discovered that a new paradigm in apoptosis regulation by TR3 in prostate cancer cells (3). We found that, in response to apoptotic stimuli, TR3 is translocated from the nucleus to the cytoplasm, where it is targeted to mitochondria, resulting in cytochrome c release and apoptosis (3). To study whether the novel nuclear-to-mitochondrial pathway for apoptosis induction by TR3 occurred in breast cancer cells, we transfected GFP–TR3 fusion construct into MDA-MB231 cells. The GFP-TR3 fusion protein was predominantly present in the nucleus in unstimulated cells. However, on treatment of cells with MM11453 and MM11384 that induce apoptosis of breast cancer cells, GFP-TR3 was associated with mitochondria (Figure 1).

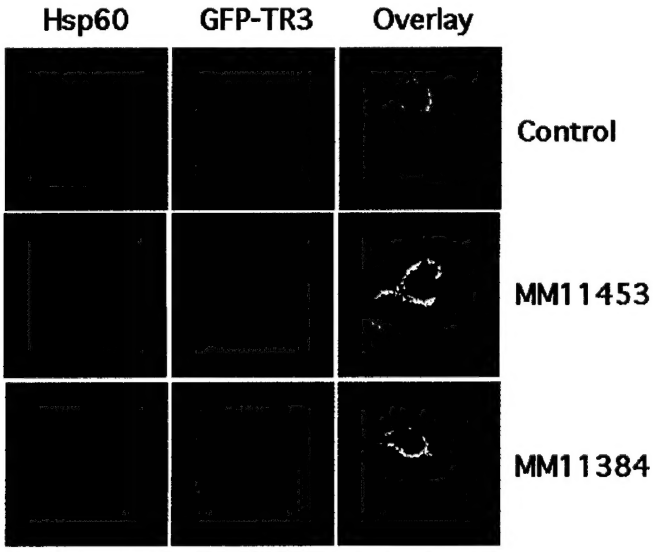

Figure 1. TR3 is targeted to mitochondria in MDA-MB231 cells in response to apoptosis inducer. GFP-TR3-transfected MDA-MB231 cells were treated with or without MM11453 (10^{-6} M) or MM11384 (10^{-6} M) for 1 hour, then immuno-stained with anti-Hsp60 antibody (Sigma) followed by Cy3-conjugated secondary antibody (Sigma) to detect mitochondria. GFP-TR3 and mitochondria (Hsp60) were visualized using confocal microscopy and the two images were overlaid (overlay). Note: upon treatment, the distribution of GFP-TR3 overlapped extensively with that of Hsp60, indicating their association.

Mitochondrial targeting of TR3 is associated with cytochrome c release. Mitochondria play a critical role in mediating many apoptotic pathways (4). Cytochrome c is exclusively present in mitochondria and is released from mitochondria in response to a variety of apoptotic stimuli. Execution of apoptosis is primarily mediated by a family of cysteine proteases called caspases. Caspase-9 is activated through its interaction with Apaf-1, which binds and becomes activated by cytochrome c, providing a link for cytochrome c release from mitochondria and the start of the caspase cascade, leading to cell death. To determine whether mitochondrial localization of TR3 was associated with apoptosis, we transfected into ZR-75-1 or MDA-MB231 cells with GFP-TR3 /ΔDBD, a mutant TR3 deleted with the DNA-binding domain. GFP-TR3/ΔDBD constititutively targeted mitochondria (Figure 2). Transfected cells were stained for mitochondria (Hsp60) and cytochrome c (cyt c), and analyzed by confocal microscopy. As shown in Figure 2, cells expressing GFP-TR3/ΔDBD displayed diffused cyt c staining, indicating the release of cyt c from mitochondria. In contrast, non-transfected cells showed punctate cyt c staining. Thus, mitochondrial localization of TR3 is associated with cyt c release.

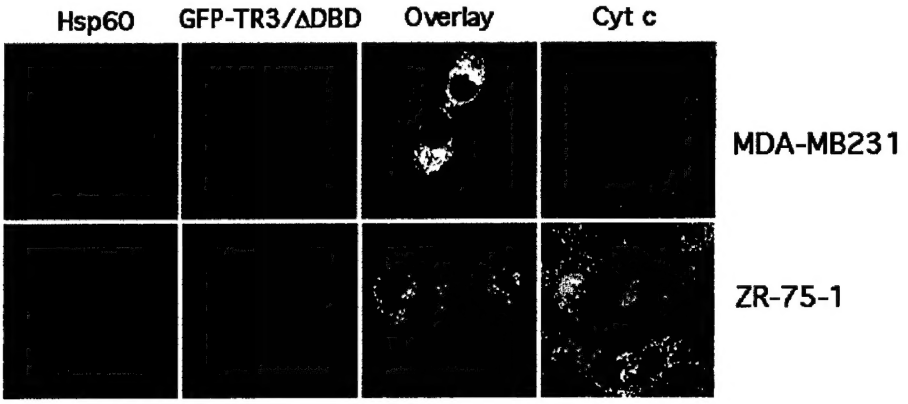

Figure 2. Mitochondrial targeting of TR3/ΔDBD is associated with cytochrome c release. GFP-TR3/ΔDBD was transiently transfected into ZR-75-1 or MDA-MB231 cells, stained for mitochondria (Hsp60) and cytochrome c (Cyt c), and analyzed by confocal microscopy.

We have also investigated whether c-myc, another immediate-early response gene, also migrated from the nucleus to mitochondria in response to apoptotic stimuli. GFP-c-myc fusion was constructed and transfected into breast cancer cells. When cells were treated with MM11384 and MM11453, however, we did not observe any mitochondrial localization of GFP-c-myc (data not shown). Our data suggest that c-myc either does not mediate apoptotic effect of MM11384 and MM11453 or it does not exert its apoptotic effect on mitochondria.

MM11453 that induces TR3 mitochondrial localization strongly promotes apoptosis of breast cancer cells. Based on our hypothesis, compounds that induce translocation of TR3 from the nucleus to mitochondria will promote apoptosis of breast cancer. To test our hypothesis, MM11453 that can induce TR3 mitochondrial targeting (Figure 1) was analyzed for its apoptotic effect in breast cancer cells (Figure 3). A loss of inner mitochondrial membrane potential or depolarization, which may signify outer membrane or permeability transition pore opening and has been suggested as causing cytochrome c release, is associated with apoptosis. The effect of MM11453 on this process was then explored using Rh123, which cells incorporate on depolarization. MM11453 increased MCF-7 and MDA-MB-231 cell Rh123 fluorescence 2.2-

and 1.9-fold, respectively (Figure 3). Thus, TR3 mitochondrial localization is associated with apoptosis of breast cancer cells in response to certain apoptotic stimuli.

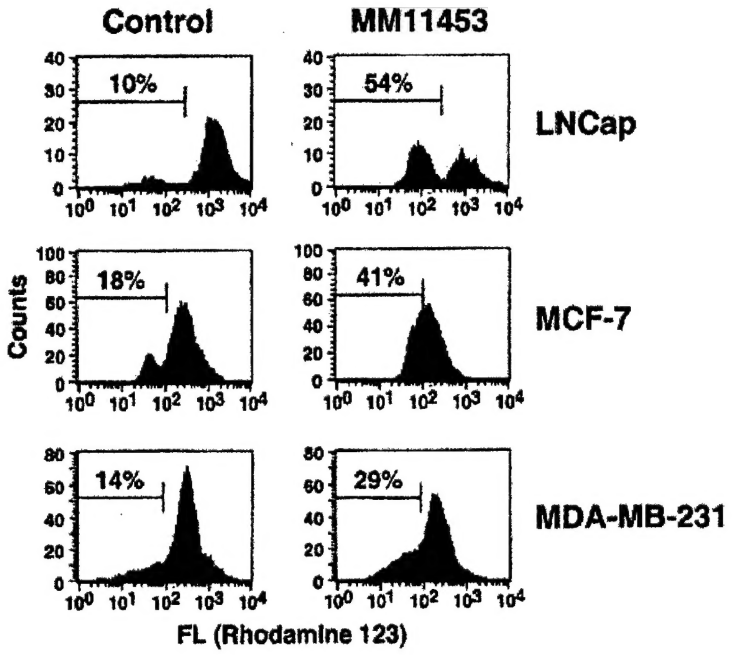

Figure 3. Effect of MM11453 on breast cancer cell mitochondrial membrane potential. MCF-7, and MDA-MB-231 cells (10,000,000) were treated with 1.0 μM MM11453 for 18 h before incubation with 5 μg/ml Rh123 for 30 min at 37°C. Rh123-fluorescing cells were scored depolarized by flow cytometry (FACScalibur system; BD Biosciences, San Jose, CA). The data shown are representative of three experiments. Rh123-fluorescencing cells are expressed as a percentage of the total.

REPORTABLE OUTCOMES

Results obtained from this concept award were included in a paper published in Cancer Research and presented in the Keystone Symposium on Nuclear Receptor Superfamily and in the FASEB Summer Conference on Retinoids.

1. Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Induction of apoptosis in cancer cells by a novel analog of 6-[3- (1-Adamantyl) –4- hydroxyphenyl} –2- naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. Cancer Research. 61:4723-4730, 2001.
2. Kolluri, S., Lin, B., Cao, X., Li, H., Lin, F., James, S., and Zhang, X-k. Nuclear orphan receptor TR3 induces apoptosis and proliferation by distinct pathways. The Keystone Symposium on Nuclear Receptor Superfamily. Snowbird, Utah, April 13-29, 2002
3. Zhang, X-k., Kolluri, S., Cao, X., Li, H., Lin, B., and Dawson, M.I. Subcellular Localization of Orphan Receptor TR3 Defines its Biological Activities. The FASEB Summer Conference on Retinoids. Tucson, Arizona, June 22-27, 2002.

CONCLUSIONS

We have conducted experiments to validate our concept that cellular localization of immediate-early genes determines their biological activities. Our results demonstrated that TR3 migrated from the nucleus to mitochondria in response to apoptotic stimuli in breast cancer cells, while c-myc retained in the nucleus under the same treatments. How c-myc exerts its opposing biological activities remains to be further determined. Our results, however, suggest that inducing TR3 mitochondrial localization may be an attractive approach to induce breast cancer cell apoptosis. However, many questions regarding how TR3 migrates from the nucleus to mitochondria and how the process is regulated remain. They need to be further investigated in order to develop novel TR3-based compounds for breast cancer treatment.

REFERENCES

1. Herschman, H. R., Primary response genes induced by growth factors and tumor promoters, *Annu Rev Biochem, 60*, 281-319 (1991).
2. Evan, G. I., and Littlewood, T. D., The role of c-myc in cell growth, *Curr Opin Genet Dev, 3*, 44-9 (1993).
3. Li, H., Kolluri, S., Gu, J., Dawson, M.I., Cao, X., Hobbs, P. Lin, B., Chen, G.-q., Lu, J.-s., Lin, F., Fontana, J. A., Reed, J. C., and Zhang, X.-k. Mitochondrial Targeting of Orphan Receptor TR3/nur77 Induces Cytochrome C Release and Apoptotic Cell Death. Science. 289: 1159-1164, 2000.
4. Green, D. R., and Reed, J. C., Mitochondria and apoptosis, *Science, 281*, 1309-12 (1998).

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[CANCER RESEARCH 61, 4723–4730, June 15, 2001]

Apoptosis Induction in Cancer Cells by a Novel Analogue of 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic Acid Lacking Retinoid Receptor Transcriptional Activation Activity[1]

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ABSTRACT

The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN) is reported to have anticancer activity *in vivo*. Induction of cell cycle arrest and apoptosis in cancer cell lines refractory to standard retinoids suggests a retinoid-independent mechanism of action for AHPN. Conformational studies suggested that binding of AHPN does not induce an unusual conformation in retinoic acid receptor (RAR) γ. The 3-chloro AHPN analogue MM11453 inhibited the growth of both retinoid-resistant (HL-60R leukemia, MDA-MB-231 breast, and H292 lung) and retinoid-sensitive (MCF-7 breast, LNCaP prostate, and H460 lung) cancer cell lines by inducing apoptosis at similar concentrations. Before apoptosis, MM11453 induced transcription factor TR3 expression and loss of mitochondrial membrane potential characteristic of apoptosis. MM11453 lacked the ability to significantly activate RARs and retinoid X receptor α to initiate $(TREpal)_2$-*tk*-CAT reporter transcription. These results, differential proteolysis-sensitivity assays, and glutathione *S*-transferase-pulldown experiments demonstrate that, unlike AHPN or the natural or standard synthetic retinoids, MM11453 does not behave as a RAR or retinoid X receptor α transcriptional agonist. These studies strongly suggest that AHPN exerts its cell cycle arrest and apoptotic activity by a signaling pathway independent of retinoid receptor activation.

INTRODUCTION

The natural RAs[3] and their synthetic analogues are being investigated as chemotherapeutic agents because they inhibit proliferation, induce apoptosis in cancer cells, and retard tumor xenograft growth (1). These standard retinoids exert their antiproliferative effects by influencing the transcriptional activity of RAR and RXR subtypes α, β, and γ (reviewed in Ref. 2). Retinoids complexed to a RXR/RAR can activate or repress gene transcription from RA response elements in the promoter of retinoid-sensitive genes. A retinoid bound to an RXR can modulate activation by other transcription factors with which it dimerizes (2). Retinoid receptor-ligand complexes also compete with other transcription factors for coactivator proteins (3, 4), whereas nonliganded dimers compete for corepressors (5).

The diversity from the six subtypes and variations in their expression patterns (2, 6–9), response element sequences, intermediary proteins, and other transcription factors (2) led to the identification of receptor-selective retinoids to enhance efficacy by reducing the systemic toxicity associated with retinoids activating all receptors (10). Receptor class and subtype-selective compounds (reviewed in Refs. 1 and 11) also provide a means for studying individual receptor-signaling pathways.

On evaluating RARγ-selective retinoids, we observed that AHPN (CD437 **[1]** in Fig. 1; Ref. 12) rapidly caused detachment of retinoid-sensitive MCF-7 breast and NIH:OVCAR-3 ovarian cancer cells (13, 14). This atypical retinoid activity extended to retinoid-resistant lines, including MDA-MB-231 breast cancer and HL-60R leukemia (13). AHPN induced cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ expression (13), G_0-G_1 cell cycle arrest (13), and apoptotic events, such as caspase activation, *gadd45* expression (15), poly(adenosyl diphosphate-ribose) polymerase cleavage, and DNA fragmentation (13). Interestingly, apoptosis occurred in the absence of functional tumor suppressor *p53* (13), the gene for which is mutated in many cancers (16). Apoptosis by AHPN and its derivatives and analogues was subsequently observed in other lines derived from tumors and their metastases (17–25).

The efficacy against retinoid-resistant cancer cells prompted studies on how AHPN induces apoptosis. To reduce complications, we conducted mechanistic studies in cells lacking functional retinoid receptors (26) and used apoptotic AHPN analogues lacking retinoid agonist transactivation activity, such as MM11453 **[2]**. MM11453 induced apoptosis by a cascade that included mitochondrial translocation of transcription factor TR3/nur77/NGFIB-II (TR3), cytochrome *c* release, caspase activation, and DNA fragmentation (27). Binding of MM11453 to RARs and RXRα did not cause the conformational changes of AHPN that led to corepressor loss and coactivator recruitment. We report here the characterization and anticancer activity of MM11453, the prototype for new nonretinoidal apoptotic agents with potential for cancer treatment.

MATERIALS AND METHODS

Retinoids. AHPN **[1]** was prepared by modifying a reported procedure (28). AHPN (MM11453) **[2]** was synthesized as follows. The biaryl bond was introduced by palladium(0)-catalyzed coupling between 3-(1-adamantyl)-4-benzyloxybenzeneboronic acid and ethyl 6-bromo-3-chloro-2-naphthalenecarboxylate [palladium(triphenylphosphine)$_4$ (Aldrich, St. Louis, MO), aqNa_2CO_3, dimethoxyethane, reflux, 6 h], followed by chromatography (6% EtOAc/hexane on silica gel) to give the benzyl-protected ethyl ester of MM11453 (66%). Benzyl group cleavage [BBr_3, CH_2Cl_2, −78°C, 2 h] to the phenol (91%) and ester group hydrolysis (aq NaOH, ethanol, 90°C, 2 h; aq HCl) gave MM11453 (95%) as a white powder, melting point 294°C–296°C (decomp.). IR (KBr): 3200, 1706, 1277, 1244, 991, 815, and 680 cm^{-1}. ^1H nuclear magnetic resonance (300 MHz, Me_2SO-d_6, δ): 1.75, 2.06, 2.17 (s, 6, adamantyl CH_2; s, 3, adamantyl CH;

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[2] To whom requests for reprints should be addressed, at The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 646-3165; Fax: (858) 646-3195; E-mail: mdawson@burnham.org.

[3] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid; aq, aqueous; GST, glutathione *S*-transferase; *tk*, thymidine kinase; CAT, chloramphenicol acetyltransferase; DPSA, differential protease sensitivity assay; TRE, thyroid hormone receptor response element; TREpal, palindromic TRE; PF, protease-resistant fragment; Hsp, heat shock protein; LBD, ligand-binding domain; Met, methionine; NCoR, nuclear receptor corepressor; Rh123, rhodamine green; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid.

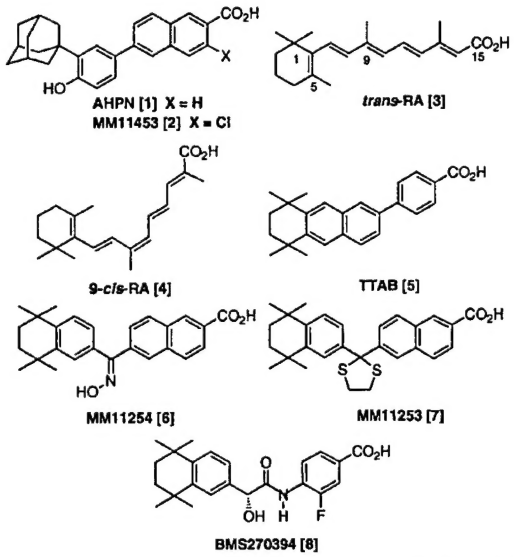

Fig. 1. AHPN [1], MM11453 [2], *trans*-RA [3], 9-*cis*-RA [4], TTAB [5], MM11254 [6], MM11253 [7], and BMS270394 [8].

s, 6, adamantyl CH_2), 6.97 (d, J = 9.0 Hz, 1, ArH-5), 7.51 (s, 1, ArH-2), 7.52 (d, J = 9.0 Hz, 1, ArH-6), 7.98 (d, J = 9.0 Hz, 1, NapH-8), 8.06 (s, 1, NapH-5), 8.25 (d, J = 8.6 Hz, 1, NapH-7), 8.27 (s, 1, NapH-4), 8.60 (s, 1, NapH-1), 9.68 (s, 1, ArOH). High-resolution mass spectrometry for $C_{27}H_{25}ClO_3$ (M^+): calculated, 432.1492; found, 432.1492. *trans*-RA [3] was purchased (Sigma Chemical Co.), as was [11,12-$^3H)_2$]9-*cis*-RA (specific activity, 43 Ci/mmol; DuPont NEN, Boston, MA). 9-*cis*-RA [4] was prepared as reported (29).

Computational Analysis. CAChe Software (Fujitsu, Beaverton, OR) was used to identify low-energy conformers within 2 kcal of the global energy minimum (MM3 force field, conjugate-gradient minimization, 30° search label variation, exclusion of ≥9 Å van der Waals interactions, and energy change <0.001 kcal/mol). Conformers were superimposed by using least-squares rigid fit of atoms corresponding to the 1, 5–9, and 15 carbon molecules of *trans*-RA.

Receptor Transcriptional Activation.[4] CV-1 cells (1,000 per well) were grown in DMEM (Irving Scientific, Santa Ana, CA) with 10% charcoal-treated FCS (Tissue Culture Biologicals, Tulare, CA) for 16–24 h before transfection, as described (30, 31). Briefly, 100 ng of $(TREpal)_2$-*tk*-CAT reporter, β-galactosidase expression vector pCH 110 (Pharmacia, Piscataway, NJ), and a RAR expression vector (or 20 ng of RXRα) were mixed with carrier DNA (pBluescript; Stratagene, La Jolla, CA) to give 1 μg of total DNA/well. CAT activity was normalized using β-galactosidase as the control. Activation after subtraction of constitutive activity is expressed relative to that of 1.0 μM *trans*-RA for RARs (100%) or 1.0 μM 9-*cis*-RA for RXRα (100%) and represents the average of three determinations.

Receptor Binding. Competitive radioligand binding on crude bacterial lysates at 0°C for 2 h used ~25 μmol of recombinant human RAR subtype or mouse RXRα-GST fusion proteins in 200 μl of binding buffer [10 mM HEPES (Sigma Chemical Co.; pH 7.8), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol] with 1–2 nM [$^3H_2$]9-*cis*-RA (43 Ci/mmol). Bound [^{3}H]9-*cis*-RA was isolated (Sephadex G-50; Pharmacia) and counted. Nonspecific [^{3}H]9-*cis*-RA binding at 1 μM nonlabeled 9-*cis*-RA generally was <10% of total label bound.

DPSA. [^{35}S]Met-labeled RARα, RARβ, RARγ, and RXRα, prepared by *in vitro* transcription/translation (32), were used in DPSA as described (33). [^{35}S]Met-labeled receptors were incubated with 0.1% ethanol alone, 1.0 μM 9-*cis*-RA, or MM11453 for 30 min at 0°C. Limited proteolysis (trypsin-tosyl phenylalanyl chloromethyl ketone; Sigma Chemical Co.) for 15 min at 22°C, followed by termination by Laemmli sample buffer and boiling and separation (10% acrylamide gel under denaturing conditions), afforded PFs for visualization by autoradiography (33, 34).

GST-Pulldown. Experiments were performed as described using GST-p300 1–450 (35) and GST-NCoR 2110–2453 (36) fusion proteins and [^{35}S]Met-labeled human RARγ.

Cell Lines and Culture. RA-resistant HL-60R cells, having a mutant RARα that does not significantly bind *trans*-RA and lacking RARβ and RARγ (26), and MDA-MB-231 cells were grown as described (20). MCF-7, LNCaP prostate, H460 and retinoid-resistant H292 lung cancer cells and Jurkat lymphoma cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Irving Scientific) with 10% charcoal-treated FCS.

Cell Growth Inhibition. HL-60R and MDA-MB-231 cells (50,000 and 100,000 per well, respectively) and 0.1–1.0 μM MM11453, AHPN, or Me_2SO alone were incubated for 24 or 120 h (72-h medium change), respectively. Results are expressed relative to Me_2SO control as mean ± SE of triplicate experiments. SEs were <10%. MCF-7, LNCaP, H292, and H460 cells (3,000 per well in 96-well plates) were treated with 1.0 μM MM11453, AHPN, *trans*-RA, or ethanol alone for 48 h before viable cell numbers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (7, 9, 27). Data shown are representative of three experiments.

Apoptosis Detection. DNA fragmentation and apoptotic bodies were assessed in at least 500 HL-60R or MDA-MB-231 cells after incubation with MM11453 for 24 or 120 h, respectively, as described above, and acridine orange staining (15). The percentage of apoptotic cells was expressed relative to the Me_2SO control as the mean ± SE of triplicate experiments. MCF-7, LNCaP, H292, H460, and Jurkat cells (3,000 per well) were treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone for 48 h, trypsinized, washed (PBS), fixed (3.7% paraformaldehyde), and stained with 4′,6-diamidino-2-phenylindole (1 μg/ml) to visualize nuclei by fluorescent microscopy (21). Cells with apoptotic nuclear morphology were scored in each 400-cell sample using a fluorescence microscope. The data are representative of three experiments.

Northern Analysis. Total RNAs were prepared (RNeasy Mini kit; Qiagen, Germany), and TR3 expression was determined on 30 μg of total RNA from each line treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone. Blotting conditions were as described (27) with β-actin expression as the control.

TR3 Mitochondrial Targeting. The expression vector for TR3/ΔDBD-GFP, a TR3 mutant lacking the DNA-binding domain fused to the green fluorescent protein expression vector, was transiently transfected into H460 cells, as described for LNCaP cells (27). Cells were treated with 1.0 μM MM11453 or ethanol alone for 6 h and then immunostained with anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Cy3-conjugated secondary antibody (Sigma Chemical Co.) to indicate mitochondria to which Hsp60 is restricted. Confocal microscopy was used to detect TR3/ΔDBD-GFP (green fluorescence) and Hsp60 (red). Images were overlaid to show colocalization.

Mitochondrial Membrane Potential Assay. LNCaP, MCF-7, and MDA-MB-231 cells (10,000,000) were treated with 1.0 μM MM11453 for 18 h before incubation with 5 μg/ml Rh123 for 30 min at 37°C. Rh123-fluorescing cells were scored depolarized by flow cytometry (FACScalibur system; BD Biosciences, San Jose, CA; Ref. 37). The data shown are representative of three experiments. Wild-type Jurkat cells or Jurkat cells stably expressing either Bcl-2 or control vector (38) were treated similarly.

RESULTS

Close-Fitting of Energy-minimized AHPN and Retinoid Conformers. Energy-minimized conformers of AHPN [1], RAR-selective *trans*-RA [3], and RAR-selective TTAB [5] (39) were overlapped. The *trans*-RA conformer was that reported in the RARγ LBD (40, 41). Three orthogonal views of these overlaps are shown in Fig. 2*A*. The major structural difference was the 1-adamantyl group of AHPN, which extended 2.2 Å more than the *trans*-RA 18-methyl group. In Fig. 2*B*, the energy-minimized conformers of RARγ-selective agonists AHPN and MM11254 [6], a (*Z*)-oxime (14) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-carbonyl)-2-naphthalenecarboxylic acid (42), are shown overlapped with RARγ-selective agonist BMS270394 [8], as found in the ligand-binding pocket of crystallized holo-RARγ (40). The

[4] Assay conducted at The Burnham Institute, La Jolla, CA, under a license agreement with Ligand Pharmaceuticals, San Diego, CA.

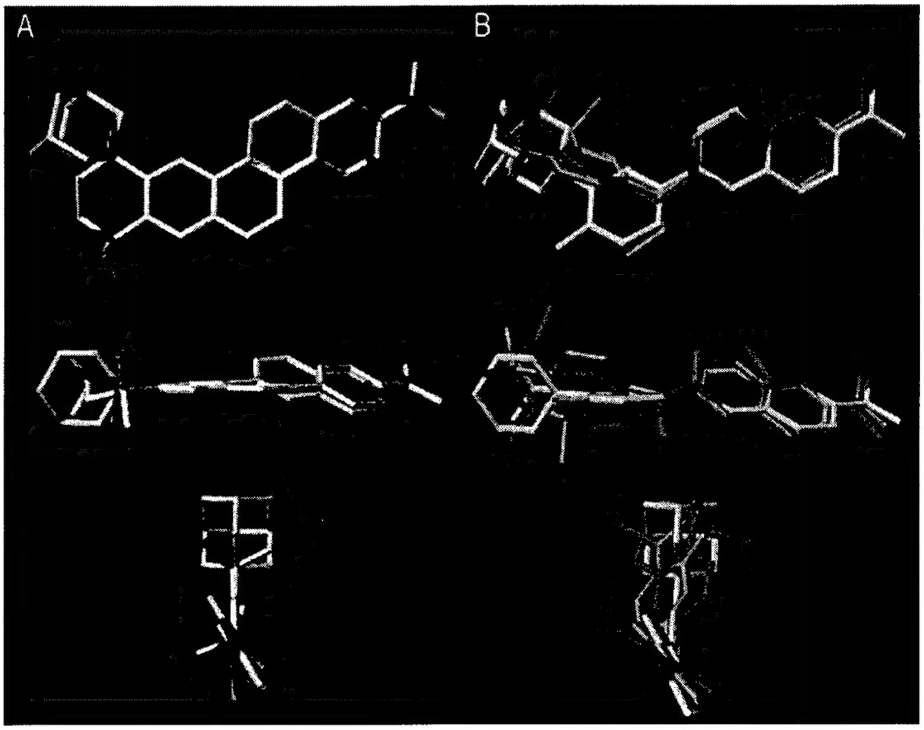

Fig. 2. Comparison of energy-minimized AHPN and retinoid conformers. Conformational analysis was performed as described in "Materials and Methods." *A*, orthogonal views of superimposed conformers of AHPN (*blue*), *trans*-RA (*red*), and TTAB (*yellow*). *B*, superimposed conformers of AHPN (*blue*), MM11254 (*green*), and BMS270394 (*magenta*).

AHPN 1-adamantyl group overlaps the saturated portion of the 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene rings of both RARγ-selective retinoids, and the AHPN phenolic oxygen is near the oxygen molecules in the oxime group of MM11254 (2.5 Å) and the bridge hydroxyl of BSM270394 (3.9 Å). Such hydroxyl groups are reported to confer RARγ selectivity by hydrogen bonding to the Met-272 sulfur molecule of RARγ (41). Placement of these overlapped conformers (Fig. 2*B*) in the RARγ ligand-binding site gives ligand O–Met-272–S distances of 4.09, 2.55, and 3.32 Å, respectively. These studies suggest that binding of AHPN to RARγ occurs in the same manner as that of standard retinoid agonists.

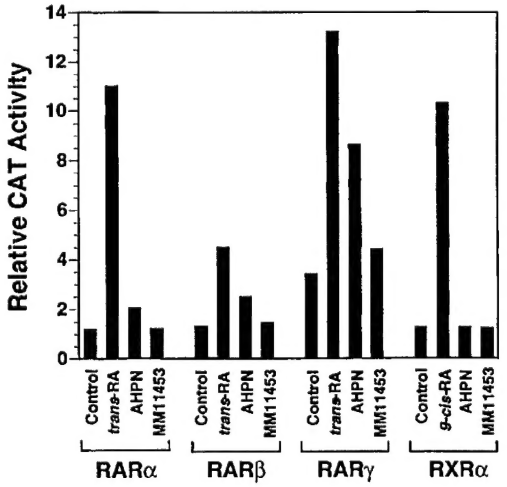

Fig. 3. Transcriptional activation of retinoid receptors by MM11453 on the $(TREpal)_2$-*tk*-CAT reporter. CV-1 cells were transiently transfected as described in "Materials and Methods," treated with 1.0 μM MM11453, AHPN, *trans*-RA, or 9-*cis*-RA, and assayed for CAT activity after 24 h. Reporter gene activation is expressed relative to 1.0 μM *trans*-RA on the RARs or 1.0 μM 9-*cis*-RA on RXRα.

MM11453 Lacked RAR Transcriptional Activation of AHPN. Although originally reported as RARγ selective on the $(TREpal)_2$-*tk*-CAT reporter in cotransfected HeLa cells (12), we observed on the $(TREpal)_2$-*tk*-CAT in CV-1 cells (4) that high RARγ selectivity occurred at 0.1 μM and below (14). At 0.5–1.0 μM, at which the natural retinoid *trans*-RA [**3**] inhibits retinoid-sensitive cancer cells, AHPN significantly activated RARβ. At 1.0 μM AHPN, reporter activation by RARα, RARβ, and RARγ was 9, 37, and 54%, respectively, of that caused by 1.0 μM *trans*-RA (Fig. 3). Unlike 1.0 μM *trans*-RA or 9-*cis*-RA, 1.0 μM MM11453 did not adequately activate any RAR subtype or RXRα to induce even modest $(TREpal)_2$-*tk*-CAT transcription. MM11453 did not activate RARα or RXRα and only activated RARβ and RARγ to 5 and 10%, respectively, of that of *trans*-RA or 13 and 19%, respectively, of that of AHPN. Thus, MM11453 is an analogue with substantially reduced capacity for RAR activation.

Retinoid Receptors Bound MM11453. Competitive ligand binding was used to determine whether MM11453 bound directly to RARs and RXRα. MM11453 at 1.0 μM displaced 61 ± 6% of $[^3H_2]$9-*cis*-RA bound to RARγ, whereas displacement from other receptors was lower [RARα (11 ± 2%), RARβ (25 ± 5%), and RXRα (18 ± 5%); Fig. 4].

MM11453 Did Not Induce an Agonist-bound RAR Conformation. DPSA on 9-*cis*-RA-bound RARα, RARβ, and RARγ produced 27-kDa PF27α, 35-kDa PF35β, and 32-kDa PF32γ, respectively (*Lane 3* in Fig. 5, *A–C*). DPSA on AHPN-bound RARs produced the same fragments (data not shown). These PFs were not observed on incubation with ethanol or MM11453 (*Lanes 2* and *4*, respectively, in Fig. 5, *A–C*). Unlike 9-*cis*-RA, neither MM11453 nor AHPN altered the proteolytic sensitivity of RXRα (data not shown). The lack of PFs from RAR-MM11453 complexes suggests that MM11453 does not

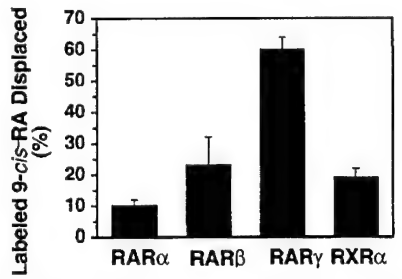

Fig. 4. Binding affinity of MM11453 to recombinant RAR and RXRα. Competition radioligand binding was conducted as described in "Materials and Methods." The data represent the means (n = 3) of the percentages of [11,12-$^3H_2$]9-*cis*-RA bound that were inhibited by 1.0 μM MM11453; *bars*, SE.

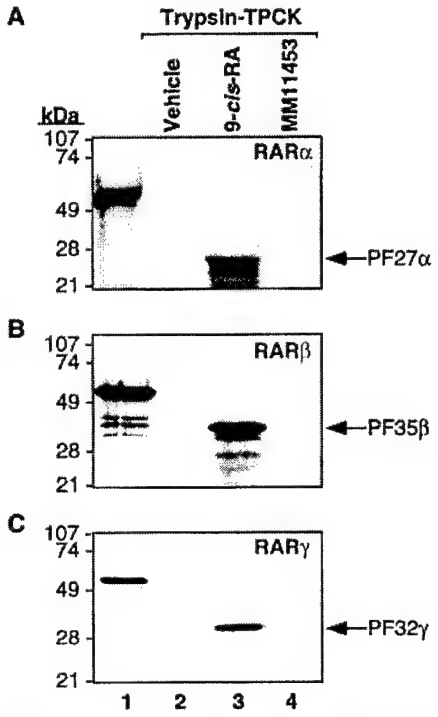

Fig. 5. MM11453 is not a RAR agonist. *A*, DPSA on [^{35}S]Met-labeled RARα in ethanol or 1 μM 9-*cis*-RA or MM11453. The migration of the 9-*cis*-RA-induced PF27α of RARα is indicated. In *B* and *C*, DPSA on RARβ and RARγ, respectively, were conducted as in *A* and "Materials and Methods." Positions of RARβ PF35β and RARγ PF35β are indicated. *Left*, marker migration (molecular mass).

promote an agonist-bound conformation. Similar to RARγ-selective antagonist MM11253,[5] MM11453 did not prevent RAR/RXR agonist 9-*cis*-RA from inducing this conformation in RARα, RARβ, or RARγ (data not shown).

MM11453 Failed to Dissociate Corepressor NCoR-RARγ *in Vitro*. GST-pulldown was used to test whether MM11453 dissociated NCoR (5) from RARγ, as 9-*cis*-RA does. As indicated (Fig. 6*A*), 9-*cis*-RA (*Lane 3*), but not MM11453 (*Lane 4*) or vehicle (*Lane 2*), disrupted the NCoR-RARγ complex.

MM11453 Failed to Recruit Coactivator p300 to RARγ. We compared the abilities of MM11453 and 9-*cis*-RA (36) to recruit p300 (43) to RARγ. Vehicle (*Lane 2* in Fig. 6*B*) or MM11453 (*Lane 4*) did not enhance p300 recruitment, whereas 9-*cis*-RA did (*Lane 3*). These findings, which agree with results on MM11453 in RARγ DPSAs (Fig. 5) and corepressor-dissociation experiments (Fig. 6*A*), confirm that MM11453 does not induce a RARγ-agonist conformation.

MM11453 Inhibited Cancer Cell Growth. Increasing evidence, including retinoid-resistant cancer cell growth inhibition (13, 27), suggests that AHPN action is independent of retinoid receptors (21, 44). Cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were conducted to show that MM11453 inhibited growth similarly. MM11453 inhibited HL-60R and MDA-MB-231 growth with IC_{50}s of 0.17 and 0.32 μM (Fig. 7, *A* and *B*), respectively, compared with AHPN values of 0.15 and 0.30 μM, respectively. Inhibition by 1.0 μM *trans*-RA was ≤5% (13). The effects of MM11453 on H460, H292, LNCaP, and Jurkat cells were then examined. As shown (Fig. 7*C*), 1.0 μM MM11453 significantly reduced growth by 70, 46, 64, and 70%, respectively, whereas 1.0 μM *trans*-RA reduced H460 growth by 15% and had no evident effect on the other lines (0–3%). Some of us reported previously that 1.0 μM AHPN for 48 h inhibited the growth of H460, H292, and LNCaP cells by 62 ± 6% (21), 53 ± 5% (21), and 100 ± 5% (25), respectively, whereas Jurkat growth was inhibited by 84% and 80 ± 3% after 24 and 96 h, respectively (22). Thus, both AHPN and MM11453 similarly retard the growth of these cell lines.

MM11453 Induced Cancer Cell Apoptosis. The MM11453 EC_{50}s for inducing nuclear fragmentation in HL-60R and MDA-MB-231 cells were 0.12 and 0.13 μM, respectively (Fig. 7, *D* and *E*), which are similar to AHPN EC_{50}s of 0.07 and 0.35 μM, respectively (13). HL-60R apoptosis inhibition by 1.0 μM MM11453 and AHPN was 82 ± 3% and 91 ± 4%, respectively, and MDA-MB-231 apoptosis

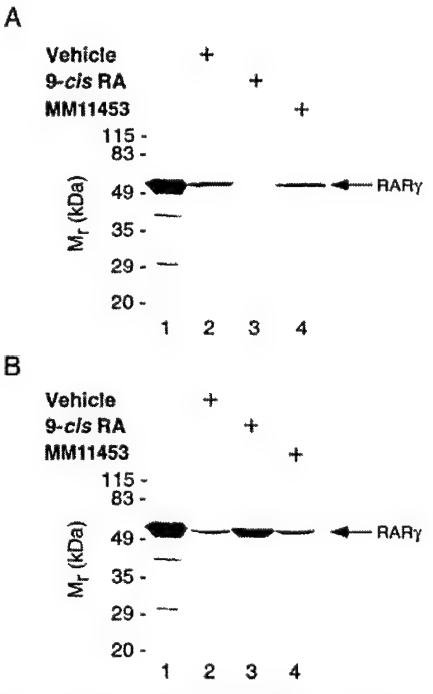

Fig. 6. MM11453 does not induce NCoR corepressor dissociation from RARγ or coactivator p300 recruitment to RARγ. *A*, NCoR-RARγ dissociation using [^{35}S]Met-RARγ and GST-NCoR 2110–2453 is as described in "Materials and Methods." Only 9-*cis*-RA induced NCoR-RARγ dissociation (*Lane 3*). In *B*, RARγ coactivator recruitment using GST-p300 (1–450) and [^{35}S]Met-RARγ is as in "Materials and Methods." Only 9-*cis*-RA enhanced binding of p300 to RARγ. *Lane 1* in *A* and *B* represents ~15% of [^{35}S]Met-RARγ. *Left*, marker migration (molecular mass).

[5] V. J. Peterson, M. I. Deinzer, M. I. Dawson, K-C. Feng, A. Fields, and M. Leid. Mass spectrometric analysis of agonist-induced retinoic acid receptor γ conformational change, unpublished results.

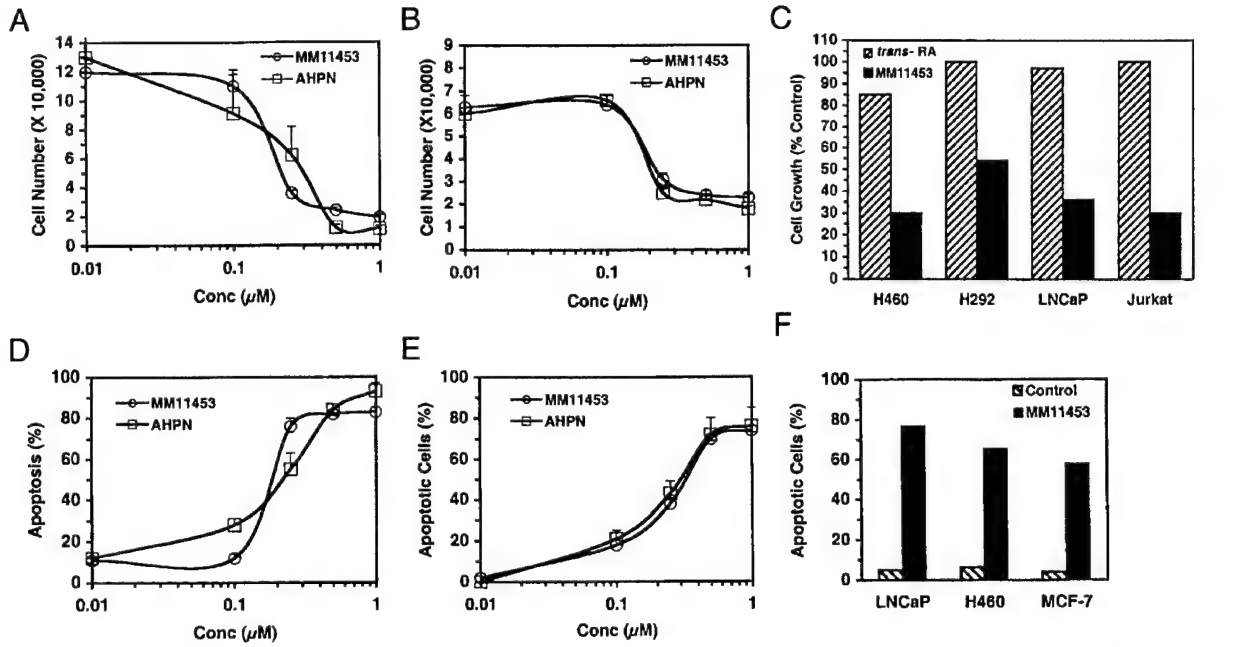

Fig. 7. MM11453 inhibits cell growth and induces apoptosis. HL-60R cells (*A* and *D*) and MDA-MB-231 cells (*B* and *E*) were treated with 10 nM to 1.0 μM MM11453, AHPN, or Me_2SO alone for 24 h and 120 h, respectively, as described in "Materials and Methods," and then harvested and counted (*A* and *B*) or assayed for apoptosis (*D* and *E*) as in "Materials and Methods." The results shown represent the means of three replicates; *bars*, SE. In *C* and *F*, H460, H292, LNCaP, and Jurkat cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 48 h before viability was determined (*C*) or treated with 1.0 μM MM11453 or ethanol alone for 48 h before nuclear morphology was analyzed (*F*) as in "Materials and Methods." The experiments shown are representative of three triplicates.

was 75 ± 1% and 76 ± 7%, respectively. Thus, both MM11453 and AHPN are similarly apoptotic in retinoid-resistant cells. MM11453 at 1.0 μM induced apoptosis in LNCaP (38%), H460 (47%), and MCF-7 (57%) cells, as demonstrated by nuclear morphological changes (Fig. 7*F*). In other experiments using these cells, 1.0 μM AHPN was found to induce 21 (21), 37, and 42% apoptosis, respectively (data not shown). Thus, both MM11453 and AHPN also induce apoptosis in retinoid-sensitive cells.

MM11453 Induced TR3 Expression. TR3 expression must be induced for AHPN to cause lung cancer cell apoptosis (21). To determine whether 1.0 μM MM11453 had this capability, H460 and LNCaP cells were treated for 6 h. MM11453 strongly induced TR3 expression, whereas *trans*-RA did not (Fig. 8).

MM11453 Induced TR3 Mitochondrial Targeting. MM11453 at 1.0 μM induced the migration of transiently expressed TR3/ΔDBD-GFP to mitochondria in H460 cells, as indicated in Fig. 9 by colocalization of GFP fluorescence with that of immunostained Hsp60. Colocalization did not occur in vehicle-alone-treated cells (data not shown). Thus, both AHPN and MM11453 induce TR3 targeting to mitochondria.

MM11453 Altered Mitochondrial Membrane Potential. We found that MM11453 induced TR3 targeting to the mitochondrial outer membrane of breast and prostate cancer cells to initiate cytochrome *c* release and apoptosis (27). A loss of inner mitochondrial membrane potential or depolarization, which may signify outer membrane or permeability transition pore opening (45) and has been suggested as causing cytochrome *c* release (45), is associated with apoptosis. The effect of MM11453 on this process was explored using Rh123, which cells incorporate on depolarization. MM11453 increased MCF-7, MDA-MB-231, and LNCaP cell Rh123 fluorescence 2.2-, 1.9-, and 5.4-fold, respectively (Fig. 10). Again, MM11453 behaves similarly to AHPN (46).

Bcl-2 Attenuated Mitochondrial Membrane Depolarization by MM11453. Because overexpression of antiapoptotic, mitochondrial membrane-surface protein Bcl-2 is reported to block cancer cell apoptosis (47), its effect on apoptosis by MM11453 was explored in Jurkat cells transfected with an expression vector containing *bcl-2* or the vector alone. In MM11453-treated nontransfected cells and MM11453-treated vector alone-transfected cells, depolarized cell numbers increased 4.8- and 5.5-fold, respectively, over that of the nontreated control, whereas cell numbers increased only 2-fold in treated cells overexpressing *bcl-2* (Fig. 11). Thus, *bcl-2* modified the effect of MM11453 on mitochondrial membranes.

DISCUSSION

AHPN induces apoptosis in cancer cell lines (13, 14, 18–22, 24). How AHPN initiates this process remains to be completely defined. A report of the RARγ selectivity of AHPN (12) led to the hypothesis of an apoptotic role for RARγ in breast cancer, melanoma, and neuroblastoma cells (17, 23, 24). To support this, RARγ transcriptionally active AHPN derivatives and analogues were also reported to inhibit growth and induce apoptosis (28, 48, 49). Other reports present data strongly suggesting an RAR-independent pathway, such as growth inhibition and apoptosis of retinoid-resistant cancer cells (13, 14, 21, 27, 42, 44, 50).[5] Our results support the latter by showing that MM11453, although unable to activate retinoid receptors on a reporter with the efficacy of standard retinoids or AHPN, strongly inhibited growth and induced apoptosis in retinoid-resistant cancer cell lines.

The near absence of RAR subtype and RXRα transcriptional activation by MM11453 was confirmed by limited proteolysis. DPSAs suggest that MM11453 is not a RAR or RXRα agonist. MM11453 did not induce a protease-resistant RARγ conformation, characteristic of binding a retinoid agonist, such as MM11254 [6], but behaved as the

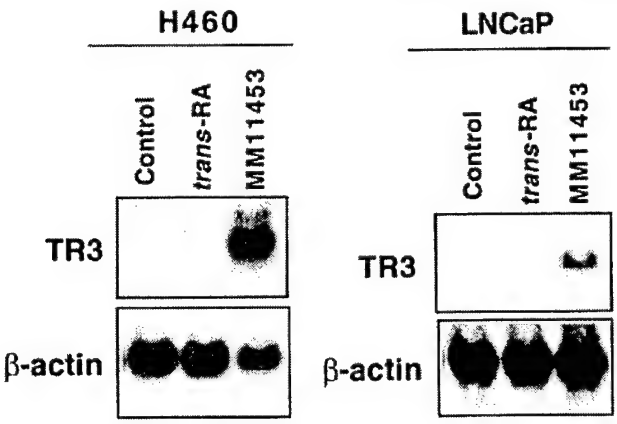

Fig. 8. MM11453 induces TR3 expression in H460 and LNCaP cells. Cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 6 h. Total RNAs were prepared and analyzed for TR3 expression by Northern blotting. Expression of β-actin was the RNA-loading control.

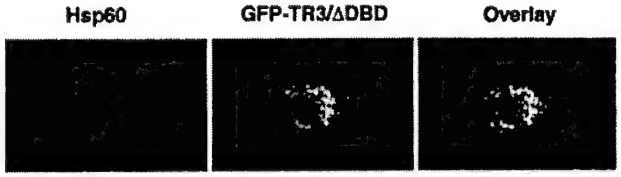

Fig. 9. MM11453 induces TR3 translocation to H460 mitochondria. Cells were transiently transfected with TR3/ΔDBD-GFP expression vector and then treated with 1.0 μM MM11453 for 6 h as in "Materials and Methods." Immunostained mitochondrial Hsp60 (*red*) and TR3/ΔDBD-GFP protein (*green*) were visualized by confocal microscopy, and images were overlaid (*Overlay*) to indicate colocalization (*yellow*).

RARγ-selective antagonist MM11253 [7], a dithiane (14, 39) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid].[5] MM11453 did not detectably dissociate NCoR from RARγ or recruit p300 to RARγ, as agonists did. Thus, the behavior of MM11453 contrasts with that of RAR-agonist AHPN (12, 14). A retinoid receptor-independent pathway for anticancer activity has precedent in the mechanism of action of *N*-(4-hydroxy)phenyl retinamide, which inhibits the growth of cancer cells that resist standard retinoids (51, 52).

DPSA (data not shown) and molecular modeling (Fig. 2) suggest that AHPN does not induce a unique conformation in the RARγ LBD that could account for apoptosis-inducing activity. RARγ on binding AHPN, *trans*-RA, or MM11254 produced the same PFs,[5] whereas 1.0 μM MM11253 [7] did not induce this conformation[5] or transcriptionally activate RARγ (14). Both transactivation and DPSA show that the bulky 1-adamantyl group of AHPN (Fig. 2*A*) did not prevent an agonist-induced RARγ conformation, and modeling shows the 1-adamantyl group occupying the same region as the tetrahydronaphthalene rings of agonists MM11254 and BMS270394 [8] (Ref. 41; Fig. 2*B*). The three hydroxyl and carboxyl oxygen molecules are also close. Thus, on the basis of the strategy used by Klaholz *et al.* (41) that the low-energy conformation of a ligand approximates its bound form, our findings suggest that pharmacophoric AHPN groups are not responsible for inducing any unique conformation in RARγ. Only the 3-chloro group *ortho* to the COOH group distinguishes MM11453 from AHPN. How the chloro group inhibits transcriptional activation remains to be determined. Both its steric and electronic properties may perturb hydrogen bonding by the COOH group or shift van der Waals contacts of RARγ LBD pendant groups, thereby preventing the conformational changes in the receptor necessary for coactivator recruitment and transcriptional activation.

The inhibition of [^{3}H]9-*cis*-RA binding to RARs by MM11453 suggests direct binding, whereas transfection indicates minimal RAR or RXRα agonism. Thus, MM11453 may function as a moderately selective RARγ antagonist. Although how RARγ antagonism or that of another RAR or RXR subtype contributes to MM11453 activity remains to be defined, the lack of growth inhibition by antagonist MM11253 (data not shown) suggests that the contribution, if any, is small. Unlike *trans*-RA, both MM11453 and AHPN strongly inhibited HL-60R, MDA-MB-231, LNCaP, and H292 cell growth and induced apoptosis. EC_{50}s for inhibiting growth in HL-60R and MDA-MB-231 cells were comparable, and their apoptotic EC_{50}s were similar (Fig. 7). These results indicate that MM11453 functions independently of RARs and RXRα and strongly suggest a similar mode of action for AHPN. Both AHPN (21) and MM11453 (Fig. 8) induced TR3 expression in H460 and LNCaP cells and TR3 mitochondrial translocation (Ref. 27 and Fig. 9, respectively) and caused inner mitochondrial membrane depolarization in MCF-7, MDA-MB-231, LNCaP, and Jurkat cells (Figs. 10 and 11). These results demonstrate that

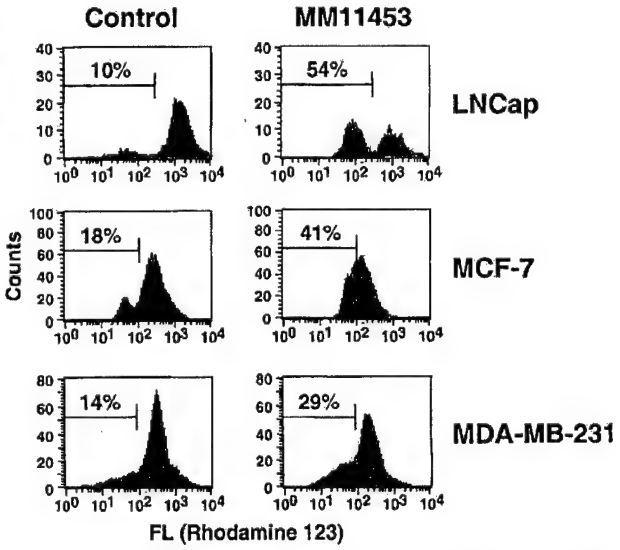

Fig. 10. Effect of MM11453 on LNCaP, MCF-7, and MDA-MB-231 mitochondrial membrane potential. Cells were treated with or without 1.0 μM MM11453 for 18 h and then with Rh123 as in "Materials and Methods." Rh123-fluorescencing cells are expressed as a percentage of the total.

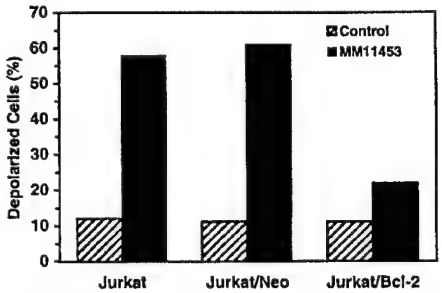

Fig. 11. Bcl-2 inhibits Jurkat mitochondrial membrane potential decrease by MM11453. Nontransfected cells stably expressing vector alone (*Jurkat/Neo*) and transfected cells stably expressing Bcl-2 (*Jurkat/Bcl-2*) were treated with 1.0 μM MM11453 or ethanol alone for 18 h and analyzed for change in mitochondrial membrane potential as in "Materials and Methods."

MM11453 retains the apoptotic properties of AHPN without behaving as a competent RARγ agonist and, thus, indicate that RARγ activation is not required for apoptotic activity. The recent report that AHPN induces apoptosis in RARγ-negative myeloma cells through a mitochondrial pathway (46) supports this conclusion. Reporter and limited proteolysis assays on MM11453 and AHPN suggest that their apoptotic activity does not involve RARα, RARβ, or RXRα activation.

Transactivation by liganded RARγ is reported to correlate with retinoid toxicity (53, 54). The lack of retinoid receptor activation activity by MM11453 suggests that toxic side effects characteristic of retinoid receptor activation (reviewed in Ref. 11) should be reduced in this class of apoptosis inducers, thereby affording more effective candidates for development as cancer chemotherapeutic agents.

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REFERENCES

1. Nagpal, S., and Chandraratna, R. A. S. Recent developments in receptor-selective receptive retinoids. Curr. Pharm. Des., *6:* 919–931, 2000.
2. Mangelsdorf, D. J., Umesono, K., and Evans, R. M. The retinoid receptors. *In:* M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), The Retinoids. Biology, Chemistry, and Medicine, pp. 319–349. New York: Raven Press, Ltd., 1994.
3. Salbert, G., Fanjul, A., Piedrafita, F. J., Lu, X-P., Kim, S. J., Tran, P., and Pfahl, M. Retinoic acid receptors and retinoid X receptor-α down-regulate the transforming growth factor-β1 promoter by antagonizing AP-1 activity. Mol. Endocrinol., *7:* 1347–1356, 1993.
4. Caelles, C., Gonzalez-Sancho, J. M., and Munoz, A. Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. Genes Dev., *11:* 3351–3364, 1997.
5. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Spoderstrom, M., and Glass, C. K. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor corepressor. Nature (Lond.), *377:* 397–404, 1995.
6. Li, X-S., Shao, Z-M., Sheikh, M. S., Eiseman, J. L., Sentz, D., Jetten, A. M., Chen, J-C., Dawson, M. I., and Fontana, J. Retinoic acid nuclear receptor β (RARβ) inhibits breast carcinoma growth and tumorigenicity. J. Cell. Physiol., *16:* 449–458, 1995.
7. Liu, Y., Lee, M-O., Wang, H-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J., and Zhang, X. RARβ mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. Mol. Cell. Biol., *16:* 1138–1149, 1996.
8. Widshwendter, M., Berger, J., Hermann, M., Muller, H. M., Amberger, A., Zeschnigk, M., Widschwendter, A., Abendstein, B., Zeimet, A. G., Daxenbichler, G., and Marth, C. Methylation and silencing of the retinoic acid receptor-β2 gene in breast cancer. J. Natl. Cancer Inst. (Bethesda), *92:* 826–832, 2000.
9. Lin, B., Chen, G., Xiao, D., Kolluri, S., Cao, X., Li, H., Kin, F., Ran, R., Su, H., and Zhang, X. Orphan receptor COUP-TF is required for RARβ induction, growth inhibition, and apoptosis by retinoic acid in cancer cells. Mol. Cell. Biol., *20:* 957–970, 2000.
10. Armstrong, R. B., Ashenfelter, K. O., Eckhoff, C., Levin, A. A., and Shapiro, S. S. General and reproductive toxicology of retinoids. *In:* M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), The Retinoids. Biology, Chemistry, and Medicine, pp. 545–572. New York: Raven Press, Ltd., 1994.
11. Dawson, M. I., Zhang, X., Hobbs, P. D., and Jong, L. Synthetic retinoids and their usefulness in biology and medicine. *In:* M. A. Livrea (ed.), Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications, pp. 161–196. Basel: Birkhäuser Verlag, 2000.
12. Bernard, B. A., Bernardon, J. M., Delescluse, C., Martin, B., Lenoir, M. C., Maignan, J., Charpentier, B., Pilgrim, W. R., Reichert, U., and Shroot, B. Identification of synthetic retinoids with selectivity for human nuclear retinoic acid receptor β. Biochem. Biophys. Res. Commun., *186:* 977–983, 1992.
13. Shao, Z. M., Dawson, M. I., Li, X. S., Rishi, A. K., Sheikh, M. S., Han, Q. X., Ordonez, J. V., Shroot, B., and Fontana, J. A. p53-independent G_0/G_1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells. Oncogene, *11:* 493–504, 1995.
14. Chao, W-R., Hobbs, P. D., Jong, L., Zhang, X-K., Zheng, Y., Wu, Q., Shroot, B., and Dawson, M. I. Effects of receptor class- and subtype-selective retinoids and an apoptosis-inducing retinoid on the adherent growth of the NIH:OVCAR-3 ovarian cancer cell line in culture. Cancer Lett., *115:* 1–7, 1997.
15. Rishi, A. K., Sun, R-J., Gao, Y., Hsu, C. K. A., Gerald, T. M., Sheikh, M. S., Dawson, M. I., Reichert, U., Shroot, B., Fornace, A. J., Jr., Brewer, G., and Fontana, J. A. Posttranscriptional regulation of the DNA damage-inducible *gadd45* gene in human breast carcinoma cells exposed to a novel retinoid. Nucleic Acids Res., *27:* 3111–3119, 1999.
16. Tarapore, P., and Fukasawa, K. p53 mutation and mitotic infidelity. Cancer Investig., *18:* 148–155, 2000.
17. Widschwendter, M., Daxenbichler, G., Culig, Z., Michel, S., Zeimet, A. G., Mörtl, M. G., Widschwendter, A., and Marth, C. Activity of retinoic acid receptor-γ selectively binding retinoids alone and in combination with interferon-γ in breast cancer cell lines. Int. J. Cancer, *71:* 497–504, 1997.
18. Oridate, N., Higuchi, M., Suzuki, S., Shroot, B., Hong, W. K., and Lotan, R. Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437). Int. J. Cancer, *70:* 484–487, 1997.
19. Hsu, S-L., Yin, S-C., Liu, M-C., Reichert, U., and Ho, W. L. Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis. Exp. Cell Res., *252:* 332–341, 1999.
20. Hsu, C. A., Rishi, A. K., Li, X. S., Gerald, T. M., Dawson, M. I., Schiffer, C., Reichert, U., Shroot, B., Poirer, G. C., and Fontana, J. A. Retinoid-induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. Blood, *89:* 4470–4479, 1997.
21. Li, Y., Lin, B., Agadir, A., Liu, R., Dawson, M. I., Reed, J. C., and Zhang, X. Molecular determinants of AHPN (CD437)-induced growth arrest and apoptosis in human lung cancer cell lines. Mol. Cell. Biol., *18:* 4719–4731, 1998.
22. Adachi, H., Adams, A., Hughes, F. M., Zhang, J., Cidlowski, J. A., and Jetten, A. M. Induction of apoptosis by the novel retinoid AHPN in human T-cell lymphoma cells involves caspase-dependent and -independent pathways. Cell Death Differ., *5:* 973–983, 1998.
23. Schadendorf, D., Worm, M., Jurgovski, K., Dippel, E., Michel, S., Charpentier, B., Bernardon, J. M., Reichert, U., and Czarnetzki, B. M. Retinoic acid receptor γ-selective retinoids exert antiproliferative effects on human melanoma cell growth *in vitro*. Int. J. Oncol., *5:* 1325–1331, 1994.
24. Meister, B., Fink, F-M., Hittmair, A., Marth, C., and Widschwendter, M. Antiproliferative activity and apoptosis induced by retinoic acid receptor-γ selectively binding retinoids in neuroblastoma. Anticancer Res., *18:* 1777–1786, 1998.
25. Liang, J-Y., Fontana, J. A., Rao, J. N., Ordonez, J. V., Dawson, M. I., Shroot, B. Wilber, J. F., and Feng, P. A synthetic retinoid CD437 induces S-phase arrest and apoptosis. Prostate, *38:* 228–236, 1999.
26. Robertson, K. A., Emami, B., and Collins, S. J. Retinoic acid-resistant HL-60R cells harbor a point mutation in the retinoic acid receptor ligand-binding domain that confers dominant negative activity. Blood, *80:* 1881–1887, 1992.
27. Li, H., Kolluri, S. K., Gu, J., Dawson, M. I., Cao, X., Hobbs, P. D., Lin, B., Chen, G. L. J., Lin, F., Xie, Z., Fontana, J. A., Reed, J. C., and Zhang, X. Cytochrome *c* release and apoptosis induced by mitochondrial targeting of orphan receptor TR3 (nur77, NGFI-B). Science (Wash. DC), *289:* 1159–1164, 2000.
28. Charpentier, B., Bernardon, J-M., Eustache, J., Millois, C., Martin, B., Michel, S., and Shroot, B. Synthesis, structure-affinity relationships and biological activities of ligands binding to retinoic acid receptor subtypes. J. Med. Chem., *38:* 4993–5006, 1995.
29. Sakashita, A., Kizaki, M., Pakkala, S., Schiller, G., Tsuruoka, N., Tomosaki, R., Cameron, J. F., Dawson, M. I., and Koeffler, H. P. 9-*cis*-Retinoic acid: effects on normal and leukemic hematopoiesis *in vitro*. Blood, *81:* 1009–1016, 1993.
30. Zhang, X., Hoffmann, B., Tran, P., Graupner, G., and Pfahl, M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature (Lond.), *355:* 441–446, 1992.
31. Wu, Q., Dawson, M. I., Zheng, Y., Hobbs, P. D., Agadir, A., Jong, L., Li, Y., Liu, R., Lin, B., and Zhang, X. Inhibition of *trans*-RA-resistant human breast cancer cell growth by retinoid X-receptor-selective retinoids. Mol. Cell. Biol., *17:* 6598–6608, 1997.
32. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J-Y., Staub, A., Garnier, J-M., Mader, S., and Chambon, P. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell, *68:* 377–395, 1992.
33. Minucci, S., Leid, M., Toyama, R., Saint-Jeannet, J-P., Peterson, V. J., Horn, V., Ishmael, J. E., Bhattacharyya, N., Dey, A., David, I. B., and Ozato, K. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid dependent gene expression. Mol. Cell. Biol., *17:* 644–655, 1997.
34. Leid, M. Ligand-induced alteration of the protease sensitivity of retinoid X receptor. J. Biol. Chem., *269:* 14175–14181, 1994.
35. Dowell, P., Ishmael, J., Avram, D., Peterson, V., Nevrivy, D., and Leid, M. p300 functions as a coactivator for the peroxisome proliferator-activated receptor α. J. Biol. Chem., *272:* 33435–33443, 1997.
36. Dowell, P., Ishmael, J. E., Avram, D., Peterson, V. J., Nevrivy, D. J., and Leid, M. Identification of nuclear receptor corepressor as a peroxisome proliferator-activated receptor α-interacting protein. J. Biol. Chem., *274:* 15901–15907, 1999.
37. Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T., and Thompson, C. B. Bcl-X_L regulates the membrane potential and volume homeostasis of mitochondria. Cell, *91:* 627–637, 1997.
38. Miyashi, T., Kitada, S., Krajewski, S., Horne, W. A., Delia, D., and Reed, J. C. Overexpression of the Bcl-2 protein increases the half-life of p21Bax. J. Biol. Chem., *270:* 26049–26052, 1995.
39. Dawson, M. I., Chao, W., Pine, P., Jong, L., Hobbs, P. D., Rudd, C., Quick, T. C., Niles, R. M., Zhang, X., Lombardo, A., Ely, K. R., Shroot, B., and Fontana, J. A. Correlation of retinoid binding affinity to RARα with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. Cancer Res., *55:* 4446–4451, 1995.

40. Klaholz, B. P., Mitschler, A., and Moras, D. Structural basis for isotype selectivity of the human retinoic acid receptor. J. Mol. Biol., *302:* 155–170, 2000.
41. Klaholz, B. P., Mitschler, A., Belema, M., Zusi, C., and Moras, D. Enantiomer discrimination illustrated by high-resolution crystal structures of the human nuclear receptor hRARγ. Proc. Natl. Acad. Sci. USA, *97:* 6322–6327, 2000.
42. Graupner, G., Malle, G., Maignan, J., Lang, G., Prunieras, M., and Pfahl, M. 6'-Substituted naphthalene-2-carboxylic acid analogs, a new class of retinoic acid receptor subtype-specific ligands. Biochem. Biophys. Res. Commun., *179:* 1554–1561, 1991.
43. Li, X-S., Rishi, A. K., Shao, Z-M., Dawson, M. I., Jong, L., Shroot, B., Reichert, U., Ordenez, J., and Fontana, J. A. Posttranscriptional regulation of $p21^{WAF-1/CIP-1}$ expression in human breast carcinoma cells. Cancer Res., *56:* 5055–5062, 1996.
44. Fontana, J. A., Dawson, M. I., Leid, M., Rishi, A. K., Zhang, Y., Hsu, C-A., Lu, J. S., Peterson, V. J., Jong, L., Hobbs, P., Chao, W-R., Shroot, B., and Reichert, U. Identification of a unique binding protein for a novel retinoid inducing cellular apoptosis. Int. J. Cancer, *86:* 474–479, 2000.
45. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. Science (Wash. DC), *281:* 1309–1312, 1998.
46. Marchetti, P., Zamzami, N., Joseph, B., Schraen-Maschke, S., Mereau-Richard, C., Costantini, P., Metivier, D., Susin, S. A., Kroemer, G., and Formstecher, P. The novel retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid can trigger apoptosis through a mitochondrial pathway independent of the nucleus. Cancer Res., *59:* 6257–6266, 1999.
47. Reed, J. C. Bcl-2 family proteins. Oncogene, *17:* 3225–3236, 1998.
48. Lu, X. P., Fanjul, A., Picard, N., Shroot, B., and Pfahl, M. A selective retinoid with high activity against an androgen-resistant prostate cancer cell type. Int. J. Cancer, *80:* 272–278, 1999.
49. Lu, X. P., Fanjul, A., Picard, N., Pfahl, M., Rungta, D., Nared-Hook, K., Carter, B., Piedrafita, J., Tang, S., Fabbrizio, E., and Pfahl, M. Novel retinoid-related molecules as apoptosis inducers and effective inhibitors of human lung cancer cells *in vivo*. Nat. Med., *3:* 686–690, 1997.
50. Sun, S. Y., Yue, P., and Lotan, R. Implications of multiple mechanisms in apoptosis induced by the synthetic retinoid CD437 in human prostate carcinoma cell. Oncogene, *19:* 4513–4522, 2000.
51. Delia, D., Aiello, A., Lombardi, L., Pelicci, P. G., Grignani, F., Grignani, F., Formelli, F., Menard, S., Costa, A., Veronesi, U., and Pierotti, M. A. *N*-(4-Hydroxyphenyl)-retinamide induces apoptosis of malignant hemopoietic cell lines including those unresponsive to retinoic acid. Cancer Res., *53:* 6036–6041, 1993.
52. Sheikh, M. S., Shao, Z-M., Li, X-S., Ordonez, J. V., Conley, B. A., Wu, S., Dawson, M. I., Han, Q-X., Chao, W., Quick, T., Niles, R. M., and Fontana, J. A. *N*-(4-Hydroxyphenyl)-retinamide (4-HPR)-mediated biological actions involve retinoid receptor-independent pathways in human breast carcinoma. Carcinogenesis (Lond.), *16:* 2477–2486, 1995.
53. Reczek, P. R., Ostrowski, J., Yu, K. L., Chen, S., Hammer, L., Roalsvig, T., Starrett, J. E., Jr., Driscoll, J. P., Whiting, G., Spinazze, P. G., Tramposch, K. M., and Mansuri, M. M. Role of retinoic acid receptor γ in the Rhino mouse and rabbit irritation models of retinoidal activity. Skin Pharmacol., *8:* 292–299, 1995.
54. Standeven, A. M., Teng, M., and Chandraratna, R. A. S. Lack of involvement of retinoic acid receptor α in retinoid-induced skin irritation in hairless mice. Toxicol. Lett., *92:* 231–240, 1997.

533 **A Novel Mechanism of the Vitamin D-Dependent Transcriptional Repression Through the Human 25-Hydroxyvitamin D3 1a-Hydroxylase nVDRE .**

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25-Hydroxyvitamin D3 1a-hydroxylase (1a-hydroxylase) in kidney primarily hydroxylates 25(OH)2D3 into 1a,25(OH)2D3, one of hormonal forms of vitamin D. Thus, it acts as a key enzyme in vitamin D biosynthesis. We have demonstrated that the negative regulation of renal 1a-hydroxylase by 1a,25(OH)2D3 and the positive regulations by PTH and calcitonin occur at transcriptional levels in intact animals and cultured cells. Subsequently, we identified a region responsible for negative regulation by 1a-hydroxylase (1a-nVDRE) in the human 1a-hydroxylase 5'-flanking region. This ligand-dependent repression in transcription requires VDR/RXR. However, VDR/RXR heterodimer exhibits no direct DNA binding to 1a -nVDRE. To clarify the molecular mechanism of the 1a,25(OH)2D3 -induced repression of transcription in the 1a -nVDRE, we screened to identify a transcriptional factor directly binding to the 1a -nVDRE, using a yeast one-hybrid system with a MCT cell cDNA expression library. The cloned factor is a member of the basic-helix loop-helix gene family, and the full-length recombinant protein directly and specifically bound 1a-nVDRE in vitro, and we named the transcriptional factor VDIR. In the absense of 1a,25(OH)2D3, VDIR activated transcription through 1a-nVDRE. The transactivation function of VDIR was further enhanced by PKA, which is the downstream signal inducer of PTH signaling. Phosphorylated VDIR by PKA recruited a co-activator, p300/CBP for gene activation. However, 1a,25(OH)2D3 binding to VDR/RXR heterodimer induced a 'protein-protein' interaction with VDIR, with recruitment of co-repressors, HDAC2/Sin3A to supress transcription. These results suggest that VDIR is a key molecule related for both positive and negative regulations of 1a-hydroxylase gene expression. Moreover, this mechanism of the vitamin D-dependent transcriptional repression for 1a-hydroxylase gene may be different from the known repression mechanisms for PTH or PTHrP genes by 1a,25(OH)2D3

534 **Induction of aromatase (CYP19) expression in breast cancer cells through a non-genomic effect of ERα**

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Aromatase plays a critical role in breast cancer development by converting androgen to estrogen. To evaluate the effect of estrogen on aromatase expression, we have performed ER transient transfection experiments using the SK-BR-3 breast cancer cell line, which is ER-negative and express aromatase. When SK-BR-3 cells were transfected with the expression plasmid pCI-ERα, but not pCI-ERβ, aromatase activity was elevated by 17β-estradiol (E2) in a dose-dependent manner. The induction could be enhanced by co-transfection with the coactivator GRIP1, and suppressed by antiestrogens such as tamoxifen and ICI182780. Using aromatase gene exon Is-specific RT-PCR, the level of promoter I.1-driven transcripts were found to be elevated in ERα transfected cells, suggesting that estrogen induces aromatase expression through the up-regulation of promoter I.1. By DNA deletion analysis of the 5'-flanking region of promoter I.1, the section between -300 and -280 upstream from exon I.1 was identified to be important for mediating E2 induction. However, a direct binding of ERα to this 20 bp region could not be demonstrated. It was found that E2 induction could be suppressed by MEK inhibitor. PD98059, and EGF receptor tyrosine kinase inhibtor, PD153035 hydrochloride. These results suggest that E2 can up-regulated aromatase expression by a non-genomic effect of ERα through cross-talk with growth factor-mediated pathways (supported by the NIH grant CA44735).

535 **Up-regulated expression of nuclear receptor coactivator GT198 in mouse embryo and human cancers.**

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Gene activation mediated by nuclear receptors and their coactivators is involved in various aspects of human physiology. In addition, genes that participate in controlling early embryonic stages have also been implicated in the development of human cancers. We have identified a nuclear receptor coactivator, GT198, that may be involved in the both processes. GT198 is a nuclear receptor coactivator that interacts with the DNA-binding domain of nuclear receptors. It potently stimulates transcription mediated by nuclear receptors including ER alpha, ER beta, TR beta, AR, GR, and PR. The expression of GT198 is highly cell-specific with high expression in spermatocytes of adult testis, but is almost absent in the remainder of adult tissues. In addition, the expression of GT198 is regulated during embryonic development. Northern analysis and immunocytochemistry studies indicate that GT198 is up-regulated around day 12 of mouse embryogenesis, with cell-type specific patterns implicating liver, developing glomeruli of kidney, brown fat, neuronal layer of retina, ventricle zone of brain, adrenal gland, and nasal epithelium. Its expression pattern suggests that GT198 might be critical in controlling development-related target genes. GT198 gene is a BRCA1 locus gene that is mapped to human chromosome 17q12-q21. Evidence has previously suggested that genes other than BRCA1 within this locus may also account for a significant number of human cancers including breast cancer. Interestingly, GT198 is up-regulated in a subset of human cancers such as breast, kidney, ovary and lung cancers, melanoma and sarcoma. The increased and altered expression of GT198 in human cancers suggest that the coactivator GT198 may participate in the regulation of oncogenesis, which might lead to the identification of GT198 as putative tumor suppressor gene. The implication of nuclear receptor coactivators in both embryonic and cancer developments provide new insight into nuclear receptor function.

536 **Nuclear Orphan Receptor TR3 induces apoptosis and proliferation by distinct pathways.**

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TR3, an immediate-early response gene and an orphan member of the steroid, thyroid hormone and retinoid receptor superfamily of transcription factors, regulates proliferation, differentiation and apoptosis. Recently, we showed that TR3 induces apoptosis through a transcriptional independent mechanism by targeting to mitochondria and releasing cytochrome c. In this study, we explored the mitogenic effects of TR3. Expression of TR3 caused proliferation of lung cancer cells. The DNA binding domain of TR3 was required for its effect on cell proliferation, in contrast to TR3-induced apoptosis, which did not require this domain. Furthermore, TR3 mutants that did not activate transcription did not cause proliferation, suggesting the requirement of TR3-induced gene expression. We studied the effect of signaling pathways on TR3 function as it is regulated by phosphorylation. Constitutively active mitogen-activated protein kinase kinase kinase 1 (MEKK1) completely inhibited TR3-induced transactivation as well as proliferation. Experiments designed to understand the mechanism of inhibition of TR3 function by MEKK1 suggested involvement of JNK. In conclusion, TR3 mediates proliferation and apoptosis through distinct mechanisms. Nuclear actions of TR3 may be required for its proliferative effects, whereas mitochondrial targeting is required for TR3-induced apoptosis.

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Subcellular Localization of Orphan Receptor TR3 Defines its Biological Activities.

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TR3 (also known as NGFI-B and nur77) is the most potent apoptosis-inducing member of the steroid/thyroid/retinoid receptor superfamily. It is also an immediate-early response gene whose expression is rapidly induced by a variety of growth stimuli. Ectopic expression of TR3 in both H460 and Calu-6 lung cancer cell lines strongly increased S/G2-phase population and BrdU staining. Analysis of TR3 mutants showed that TR3 DNA binding and transactivation are required for its mitogenic effect. The mitogenic effect of TR3 could be suppressed by MEKK1 through its activation of Jun N-terminal kinase (JNK). JNK efficiently phosphorylated TR3 in immunocomplex kinase assay, resulting in loss of TR3 DNA binding and transactivation function. Paradoxically, TR3 expression was also required for apoptosis of certain cancer cells induced by a new class of synthetic retinoids related to AHPN (CD437) (6-[3-(1-adamantyl)–4-hydroxyphenyl]-2-naphthalenecarboxylic acid). However, transcriptional activity or DNA binding of TR3 was not required for its apoptotic effect. Instead, TR3, in response to AHPN and other apoptotic stimuli, translocated from the nucleus to the cytoplasm where it was targeted to mitochondria to induce cytochrome c release. Mutational analysis showed that sequences in both C-terminus and N-terminus of TR3 were critical determinants that mandate its cytoplasmic localization, mitochondrial targeting and induction of cytochrome c release, whereas the central sequences encompassing the DNA binding domain were dispensable. The C-terminal domain of TR3 was required for nuclear export of TR3 through its heterodimerization with retinoid X receptor (RXR). Together, our results demonstrate that TR3 confers the growth advantage of cancer cells in the nucleus through its transactivation function, whereas it exerts its apoptotic effects in mitochondria by inducing cytochrome c release.